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# Higher Prevalence of Borna Disease Virus Infection in Blood Donors Living Near Thoroughbred Horse Farms

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It is believed that Borna disease virus (BDV), an etiological agent of progressive polioencephalomyelitis in horses and sheep, is closely associated with psychiatric disorders in humans since the prevalence of BDV is higher in psychiatric patients than in blood donors. We investigated whether or not BDVs in humans are derived from infected domestic animals, by characterizing the BDVs in blood donors and horses derived from the same region of Hokkaido island, Japan. The seroprevalences (2.6 to 14.8%) of BDV were significantly higher in the blood donors from four regions where most horse farms are concentrated, compared with only 1% in the blood donors from Sapporo, the largest city in Hokkaido. BDV RNA was also detected in peripheral blood mononuclear cells from most of the seropositive horses and blood donors by nested reverse transcriptase-polymerase chain reaction. These findings support that BDV may be horizontally transmitted, at least in part, from infected horses to humans. ***J. Med. Virol. 52:330–335, 1997.*** 

**KEY WORDS:** Borna disease virus; blood donor; horse; horizontal transmission; RT-PCR; peripheral blood mononuclear cells

## INTRODUCTION

Borna disease (BD) is a transmissible, progressive polioencephalomyelitis of horses and sheep. The causative agent, BD virus (BDV), is a neurotropic, yet unclassified, nonsegmented, negative-sense, single-strand RNA virus [de la Torre, 1994; Schneemann et al., 1995]. Although neurological symptoms characteristic of BD in horses have been recognized mainly in Germany [Ludwig et al., 1993], infection has also been demonstrated even in horses that were either clinically normal or were pathologically diagnosed with a disease unrelated to BD [Kao et al., 1993; Richt et al., 1993a; Nakamura et al., 1995; Bahmani et al., 1996].

Accumulated sero-epidemiological data suggest the association of BDV with specific psychiatric patients [Rott et al., 1985; Bode et al., 1988, 1992, 1993, 1995; Richt et al., 1993b; Kishi et al., 1995b; Sauder et al., 1996] or in patients with chronic fatigue syndrome [Nakaya et al., 1996; Kitani et al., 1996]. Recent molecular epidemiological studies by nested reverse transcriptase-polymerase chain reaction (RT-PCR) to detect BDV RNA in peripheral blood mononuclear cells (PBMCs) confirmed these conclusions [Bode et al., 1995; Kishi et al., 1995b; Nakaya et al., 1996; Kitani et al., 1996; Sauder et al., 1996]. However, it has also been reported that healthy individuals contain serum antibodies to BDV [Bode et al., 1988, 1992, 1993; Richt et al., 1993b; Kishi et al., 1995a; Sauder et al., 1996] or BDV RNA in PBMCs [Kishi et al., 1995a; Sauder et al., 1996], although the prevalences were very low compared with those in patients with psychiatric disorders or chronic fatigue syndrome. Thus, BDV infection among people living in areas where they may be in contact with BDV-sensitive animals seems widespread in a subclinical form with a long incubation period.

Our previous study revealed that the horses from one

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TABLE I. Features of Blood Donors and Horses

| Source | | Average age in year[a] | Males | Females |
|---|---|---|---|---|
| BDV seroprevalence: | | | | |
| Blood donors | Monbetsu | 35.3 (18–59) | 33[b] | — |
| | | 40.8 (19–63) | — | 75[b] |
| | Shizunai | 33.6 (16–60) | 53 | — |
| | | 39.3 (18–60) | — | 59 |
| | Urakawa | 39.3 (19–64) | 75 | — |
| | | 35.6 (18–64) | — | 55 |
| | Shintoku | 45.8 (21–64) | 49 | — |
| | | 40.6 (19–64) | — | 29 |
| | Sapporo | 37.9 (17–66) | 71 | — |
| | | 37.8 (17–61) | — | 29 |
| BDV RNA in PBMCs: | | | | |
| Blood donors | Monbetsu | 37.7 (17–60) | 21 | — |
| | | 37.6 (17–60) | — | 36 |
| Horses | Monbetsu | 9.6 (2–17) | 21 | — |
| | | 9.3 (2–16) | — | 33 |

[a]Parentheses include age range.
[b]Total number of donors or horses.

farm in Hokkaido island of Japan were positive for BDV at a considerably high rate [Nakamura et al., 1995]. In the present study, we examined the prevalence of BDV infection in blood donors derived from several regions in this island, where most of the horses in Japan are bred. We compared the sequences of the RT-PCR products in samples from blood donors and horses living in the same region.

## MATERIALS AND METHODS

### Blood Donors and Horses

We studied BDV seroprevalence in a total of 428 blood donors in four regions (Monbetsu, Shizunai, Urakawa and Shintoku) in the northernmost island of Japan (Hokkaido), where thoroughbred race horses are bred (Table I). The control group consisted of blood donors was from Sapporo, the largest city in Hokkaido (Table I).

The prevalence of BDV RNA was determined in PBMCs. The ethylenediamine tetraacetic acid (EDTA)-treated blood samples were obtained from horses and donors in Monbetsu (Table I). The PBMCs from each sample were isolated by centrifugation using Ficoll-Paque (density 1.077).

### BDV and Cells

Controls were MDCK cells uninfected or persistently infected with horse-derived BDV (MDCK/BDV) [Herzog and Rott, 1980].

### Detection of Anti-BDV Antibodies

Anti-BDV antibodies in the plasma obtained from blood donors were examined by enzyme-linked immunosorbent assay (ELISA) as described [Kitani et al., 1996; Auwanit et al., 1996] and by immunoblotting as described [Kishi et al., 1995b]. The recombinant full-length p24 [Kishi et al., 1995b] and p40 [Bahmani et al., 1996] fusion proteins with glutathione S-transferase (GST) expressed in *E. coli* were used as BDV antigens in the assays. GST-p24, GST-p40, and GST alone as a negative control were used after purification by glutathione Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) column chromatography. ELISA microplates were coated with the purified antigens at 10 μg/ml, then reacted with a 100-fold dilution of sera or plasma. We used control human plasma that was confirmed positive or negative to BDV GST-p24 by immunoblotting [Kishi et al., 1995a], in all ELISA microplates. For immunoblotting, the GST-p24 and GST were separated by SDS-PAGE with a 15% polyacrylamide gel and were blotted onto a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA). Then, the specific reactions with a 50-fold dilution of sera were detected by Immunostaining HRP-1000 kit (Konica, Tokyo, Japan). The molecular weight values of the proteins in immunoblotting were calculated by comparing their mobilities with those of marker proteins in a calibration kit (Bio-Rad Laboratories, Richmond, CA).

### Detection of BDV RNA in PBMCs

The PBMCs were prepared from the EDTA-treated blood which were examined for antibodies to BDV. Total RNA from each PBMC sample was prepared using an RNA extraction kit (ISOGEN, Nippon Gene Co., Tokyo, Japan). To detect BDV-specific RNA, the extracted RNA (1 μg) was amplified by nested RT-PCR, under the described conditions [Kishi et al., 1995b], to obtain fragments of the p24 coding region. Following two sets of primers were used: 1st PCR, nucleotides 1387 to 1405 and 1865 to 1847; and 2nd PCR, nucleotides 1443 to 1461 and 1834 to 1816. RT-PCR consisting of reverse transcription and amplification of the viral cDNA, proceeded according to the protocol described for the EZ *rTth* RNA PCR kit (Perkin-Elmer Corporation, Norwalk, CT). As a control, representative RNA (1 μg) samples were reverse-transcribed before amplification, by STRATASCRIPT II (Stratagene, La Jolla, CA) using oligo $d(T)_{16}$ as a primer, as described previously [Bahmani et al., 1996]. The cDNA products were similarly

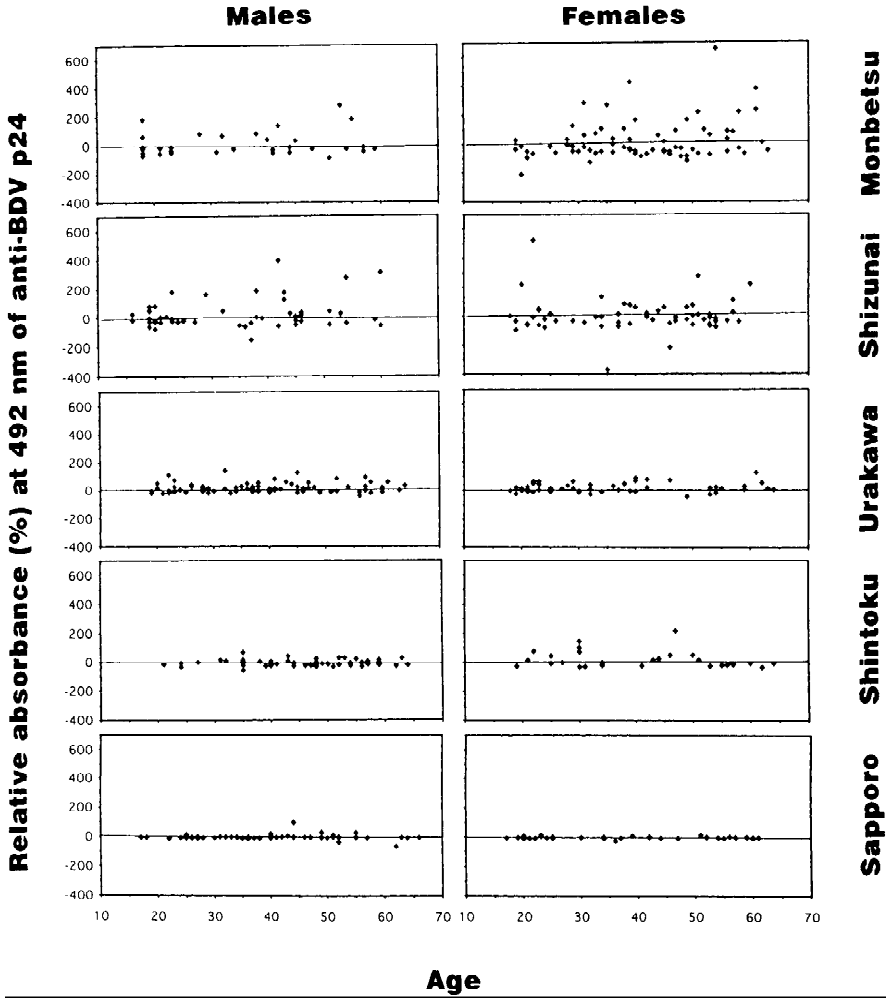


Fig. 1. Comparative seroprevalences of anti-BDV antibodies among blood donors from five regions in Hokkaido. The serum samples from a total of 528 blood donors (33 males and 75 females from Monbetsu; 53 males and 59 females from Shizunai; 75 males and 55 females from Urakawa; 49 males and 29 females from Shintoku; and 71 males and 29 females from Sapporo) were analyzed by ELISA using BDV-p24 protein as a BDV antigen. GST alone was used as a negative control antigen. The results of males and females are shown in the left and right columns, respectively as relative absorbance at 492 nm, calculated as described in the text.

amplified by nested PCR using the same two sets of primers as above. The final products were separated by 1.5% agarose gel electrophoresis, blotted onto a nylon membrane, then Southern-hybridized using following four $^{32}$P-labeled synthetic oligonucleotides as probes for this region: sense nucleotides 1462–1485, 1485–1507, and 1637–1658, and the antisense nucleotide 1811–1791. The numbers for BDV nucleotide sequences described here correspond to the reported numbering scheme for BDV isolates [Cubitt et al., 1994; Schneider et al., 1994].

### Cloning and Sequencing

Specific PCR products were cloned into a pUC18 plasmid vector (Pharmacia Biotech AB) and sequenced using the Dye Primer Cycle Sequencing kit (Applied Biosystems, Foster City, CA) in a 373A DNA Sequencer. The nucleotide sequences were analyzed by GENETYX-MAC (Software Development Co., Ltd., Tokyo, Japan).

## RESULTS

We examined the presence of anti-BDV p24 antibodies in a total of 428 serum samples from Monbetsu, Shizunai, Urakawa, Shintoku, as well as in 100 samples from Sapporo (Table I). Antibodies were detected by ELISA using GST-p24 and GST alone as a control (Fig. 1). The seroprevalences in all the regions were significantly higher than that in Sapporo, where only one sample was confirmed positive by immunoblotting (data not shown). The absorption of this sample at 492 nm was significantly higher than that of all other samples in Sapporo. Therefore, all the values of samples are expressed as relative absorbance compared with this serum as a positive standard. The negative standard was one sample from Sapporo that was also confirmed by immunoblotting (data not shown). The relative absorbance in each sample was calculated by subtracting the absorption value with GST alone, from that with GST-p24, compared with

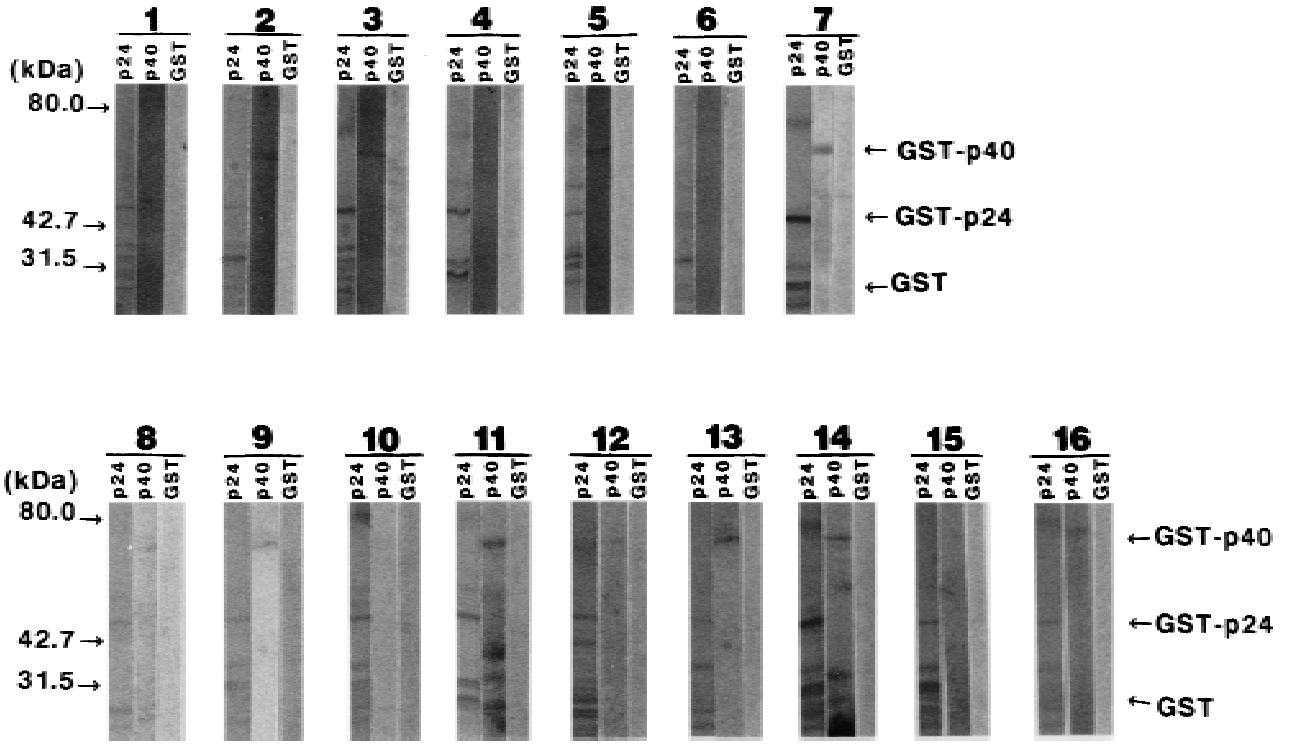


Fig. 2. Detection of BDV-specific antibodies in the plasma obtained from horses and blood donors in Monbetsu by immunoblotting. BDV-positive subjects were selected from 57 blood donors and 54 horses in Monbetsu by ELISA as in Figure 1. The ELISA-positive seven blood donors (**lane 1,** MnH1; **2,** MnH2; **3,** MnH4; **4,** MnH33; **5,** MnH44; **6,** MnH45; and **7,** MnH54) and 9 horses (**8,** MnE5; **9,** MnE7; **10,** MnE15; **11,** MnE31; **12,** MnE32; **13,** MnE35; **14,** MnE45; **15,** MnE50; and **16,** MnE53) were examined for anti-BDV antibodies by immunoblotting. The plasma (at a 100-fold dilution) were reacted with the PVDF membranes blotted with GST-p24 (approximately 51 kDa), GST-p40 (approximately 67 kDa), and GST alone (approximately 27 kDa) as a control. The molecular weight values (MW) are indicated.

those of the positive (100%) and negative (0%) control sera. Thus, the samples with values over 100% were determined as positive.

In contrast to 1% in Sapporo, BDV seroprevalence in other regions was significantly higher, being 14.8% in Monbetsu; 12.5% in Shizunai; 3.1% in Urakawa; and 2.6% in Shintoku (Fig. 1). Several random ELISA-positive sera were also confirmed by immunoblotting (data not shown). Seroprevalence slightly differed between males and females in all these regions: 12.1% and 16% in Monbetsu; 15.1% and 10.2% in Shizunai; 4.0% and 1.8% in Urakawa; 0% and 6.9% in Shintoku; and 1.4% and 0% in Sapporo (Fig. 1).

Next, we compared BDV prevalences between newly prepared blood samples from 57 blood donors in Monbetsu and from 54 thoroughbred race horses bred in this region (Table I). First, ELISA under the same conditions used to generate the results shown in Figure 1 showed prevalences of 12.3% (7/57) in blood donors and 16.7% (9/54) in horses, although the values were very low in most human and in some horse samples (data not shown). Immunoblotting showed that among seven ELISA-positive blood donors (MnH1, MnH2, MnH4, MnH33, MnH44, MnH45, and MnH54) and nine ELISA-positive (MnE5, MnE7, MnE15, MnE31, MnE32, MnE35, MnE45, MnE50, and MnE53) horses, all were positive for anti-BDV p24, while four and seven were positive for p40 antibodies (Fig. 2). Next, ELISA-positive seven blood donors and nine horses were examined for BDV RNA at the p24 region in their PBMCs by nested RT-PCR (Fig. 3). The results showed that seven of nine horse samples amplified a discrete p24 fragment of 392 bp that corresponded to the size of MDCK/BDV. Similar results were obtained from four of seven blood donors. However, the PBMC sample from one blood donor (MnH45) was faintly positive after RT-PCR, being detectable only after Southern blotting (data not shown). The samples from the other three blood donors and all seven horses showed clear positive signals by both ethidium bromide staining (Fig. 3) and Southern blot hybridization (data not shown). The RT-dependence of amplification products was confirmed by nested PCR without reverse transcription step (Fig. 3). These results on anti-p24 and p40 antibodies and p24 RNA were summarized in Table II. Thus, the individuals positive for BDV antibodies were not always positive for BDV RNA in PBMCs, especially in the blood donors.

We cloned these PCR products into pUC18, then compared them to sequences between BDVs derived from the blood donors and horses in Monbetsu. We did not obtain a cDNA clone from the PCR product derived from the MnH45 sample. Sequencing of randomly se-

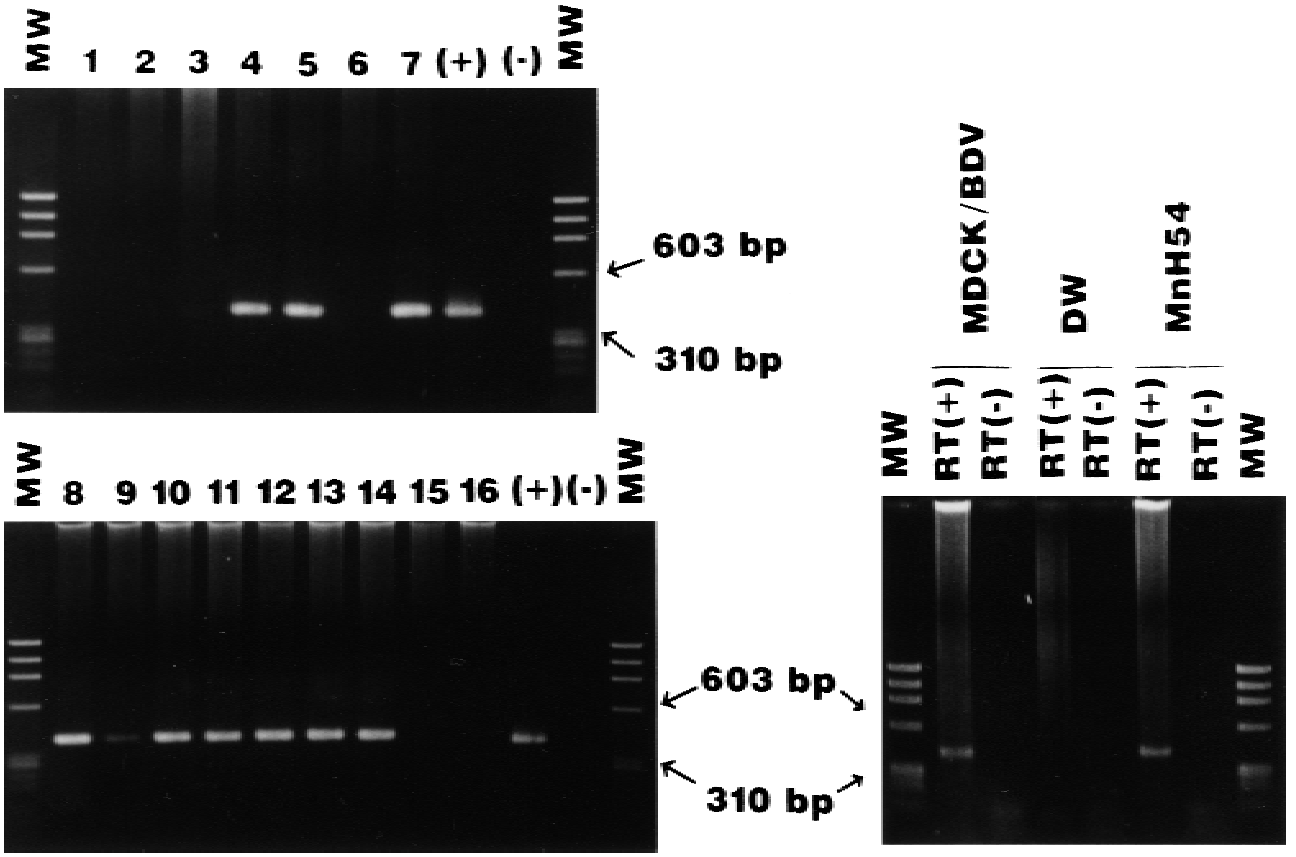


Fig. 3. Nested RT-PCR amplification of BDV RNA at the p24 region in PBMCs from the blood donors and horses in Monbetsu. The same ELISA-positive seven blood donors (**lane 1,** MnH1; **2,** MnH2; **3,** MnH4; **4,** MnH33; **5,** MnH44; **6,** MnH45; and **7,** MnH54) and nine horses (**8,** MnE5; **9,** MnE7; **10,** MnE15; **11,** MnE31; **12,** MnE32; **13,** MnE35; **14,** MnE45; **15,** MnE50; and **16,** MnE53), as for Figure 2, were examined for BDV RNA in PBMCs by nested RT-PCR using EZ *rTth* PCR kit. Total cellular RNA extracted from the PBMCs were amplified by nested RT-PCR at the p24 region of BDV. The ethidium bromide-staining profiles are shown. Positive and negative controls were the RNA fractions from MDCK/BDV (+) and MDCK (–), respectively. The RNAs from MDCK/BDV and MnH54 or distilled water (DW) in place of RNA were amplified by nested PCR with [RT(+)] and without [RT(–)] reverse transcription step. MW indicates size markers (ø × 174 DNA/*Hae* III fragments).

lected nine and 19 cDNA clones from three blood donors (MnH33, MnH44, and MnH54) and seven horses (MnE5, MnE7, MnE15, MnE31, MnE32, MnE35, and MnE45), respectively, showed that BDVs derived from horses and blood donors were essentially closely related (data not shown).

## DISCUSSION

About 90% of the thoroughbred race horses in Japan are bred in Hokkaido. In this work, we focused on the BDV prevalence of blood donors living in the four Hokkaido regions where most of the horse farms are concentrated. The seroprevalence of BDV infection by ELSA with p24 protein was significantly higher in blood donors from all regions examined (Fig. 1). These prevalences were considerably higher than that in blood donors from Sapporo, the biggest city in Hokkaido (Fig. 1) or from Tokyo (a megalopolis in Honshu, another of the islands of Japan) [Kishi et al., 1995a]. Thus, BDV may be transmitted, at least in part, from horses to humans. Similar horizontal transmission from ostriches to humans has been suggested by a seroepidemiological study of BDV infection among three groups in Israel [Malkinson et al., 1993; Weisman et al., 1994].

Further, we confirmed the high BDV prevalences in horses and blood donors derived from the same region, Monbetsu. Most of ELISA-positive blood donors and horses were positive for anti-p24 and -p40 antibodies by immunoblotting (Fig. 2) and also positive for BDV p24 RNA in PBMCs by RT-PCR (Fig. 3). However, the blood donors positive for BDV antibodies were not always positive for BDV RNA in PBMCs (Table II). These observations suggest that the BDV in these individuals might be present in the tissue cells other than PBMCs, although we can not rule out the possibility that the sensitivity of our method to detect BDV RNA is lower than the amounts in the PBMCs. Sequencing of the PCR product revealed the closely related BDV between horses and blood donors in this region (data not shown). Further comparative studies on the BDV whole genomes from blood donors and horses in Mon-

TABLE II. Summarized Results on Anti-BDV p24 and p40 Antibodies and BDV p24 RNA in PBMCs From Blood Donors and Horses in Monbetsu

| Subject | Anti-p24 | Anti-p40 | p24 RNA |
|---|---|---|---|
| Blood donors: | | | |
| MnH1 | + | − | − |
| MnH2 | + | + | − |
| MnH4 | + | + | − |
| MnH33 | + | − | + |
| MnH44 | + | + | + |
| MnH45 | + | − | + |
| MnH54 | + | + | + |
| Horses: | | | |
| MnE5 | + | + | + |
| MnE7 | + | + | + |
| MnE15 | + | − | + |
| MnE31 | + | + | + |
| MnE32 | + | + | + |
| MnE35 | + | + | + |
| MnE45 | + | + | + |
| MnE50 | + | − | − |
| MnE53 | + | + | − |

betsu will be required to confirm the evidence for horizontal transmission of BDV from infected animals to humans.

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